Investigation of the Proposed Interdomain Ribose Zipper in Hairpin Ribozyme Cleavage Using 2′-Modified Nucleosides

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ABSTRACT: The hairpin ribozyme achieves catalytic cleavage through interaction of essential nucleotides located in two distinct helical domains that include internal loops. Initial docking of the two domains is ion dependent and appears to be followed by a structural rearrangement that allows the ribozyme to achieve a catalytically active state that can undergo cleavage. The proposed structural rearrangement may also be ion dependent and is now of increased importance due to recent evidence that docking is not rate limiting and that metal ions are unlikely to be involved in the chemical cleavage step. An initial structural model of the docked hairpin ribozyme included a proposal for a ribose zipper motif that involves two pairs of hydroxyl groups at A_{10} and G_{11} in domain A pairing with C_{25} and A_{24} in domain B, respectively. We have used a chemical functional group substitution technique to study whether this proposed ribose zipper is likely to be present in the active, conformationally rearranged ribozyme that is fit for cleavage. We have chemically synthesized a series of individually modified hairpin ribozymes containing 2′-analogues of nucleosides, that include 2′-deoxy and 2′-deoxy-2′-fluoro at each of the four nucleoside positions, 2′-amino-2′-deoxy, 2′-deoxy-2′-thio, and 2′-arabino at position C_{25}, and 2′-oxyamino at position A_{10}, as well as some double substitutions, and we studied their cleavage rates under both single- and multiple-turnover conditions. We conclude that at least some of the hydrogen-bonding interactions in the ribose zipper motif, either as originally proposed or in a recently suggested structural variation, are unlikely to be present in the active rearranged form of the ribozyme that undergoes cleavage. Instead, we provide strong evidence for a very precise conformational positioning for the residue C_{25} in the active hairpin. A precise conformational requirement would be expected for C_{25} if it rearranges to form a base-triple with A_9 and the essential residue neighboring the cleavage site G_{+1}, as recently proposed by another laboratory. Our results provide further support for conformational rearrangement as an important step in hairpin ribozyme cleavage.

The important roles that RNA structure and ligand interactions play in ribozyme activity have become widely recognized in recent years. The hairpin ribozyme has proved particularly interesting in that it has provided structural and catalytic paradigms distinct from those of other small catalytic RNA motifs of the same family in which 5′-phosphate and 2′,3′-cyclic phosphate termini are generated during a cleavage reaction (reviewed in refs *1*−*4*). The hairpin ribozyme occurs as a 4-helix junction in the satellite RNA of a number of plant viruses, such as tobacco ringspot virus, and has been found to be both a nuclease and an efficient ligase, each activity being thought to require the presence of divalent metal ion. In its minimal form for cleavage, the hairpin consists of just two helical domains (Figures 1 and 2), one of which contains the cleavage site 5′ to an essential G residue. Each helical domain A and B is interrupted by an internal loop. No high-resolution crystallographic or NMR models are yet available for the minimal hairpin. However, recent NMR structures of the two individual domains have demonstrated that each domain contains a number of unusual cross-strand non-Watson−Crick base pairs within the internal loops (*5*, *6*). The loops nevertheless retain a high degree of flexibility.

A range of data generated from mutagenesis, cross-linking, and chemical structure-probing have pointed to the need for the two domains to dock and adopt a noncoaxial structure in which internal loops A and B come into close proximity (*7*−*10*). Additional and convincing evidence for such docking has been provided by FRET[1] experiments, both in the context of the 4-way helical junction and the minimal 2-domain hairpin ribozyme (*11*, *12*). These results showed that docking is a faster process (ca. 0.5 min^{-1}) than the

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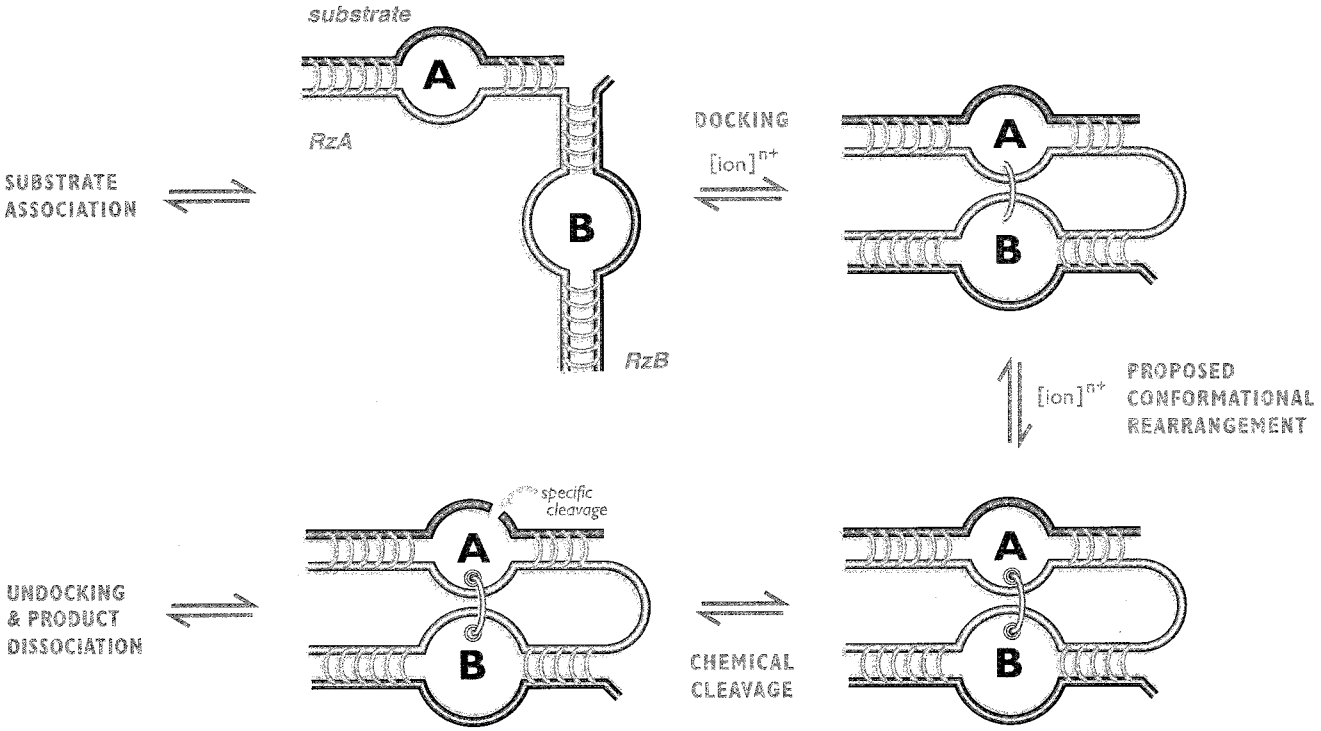

FIGURE 1: Schematic representation of the steps in the kinetic pathway for the cleavage activity of the hairpin ribozyme. We have omitted possible unproductive stacked conformations of the hairpin that may compete with docking in some cases. The proposed conformational rearrangement that precedes chemical cleavage is indicated by ball-and-stick symbols.

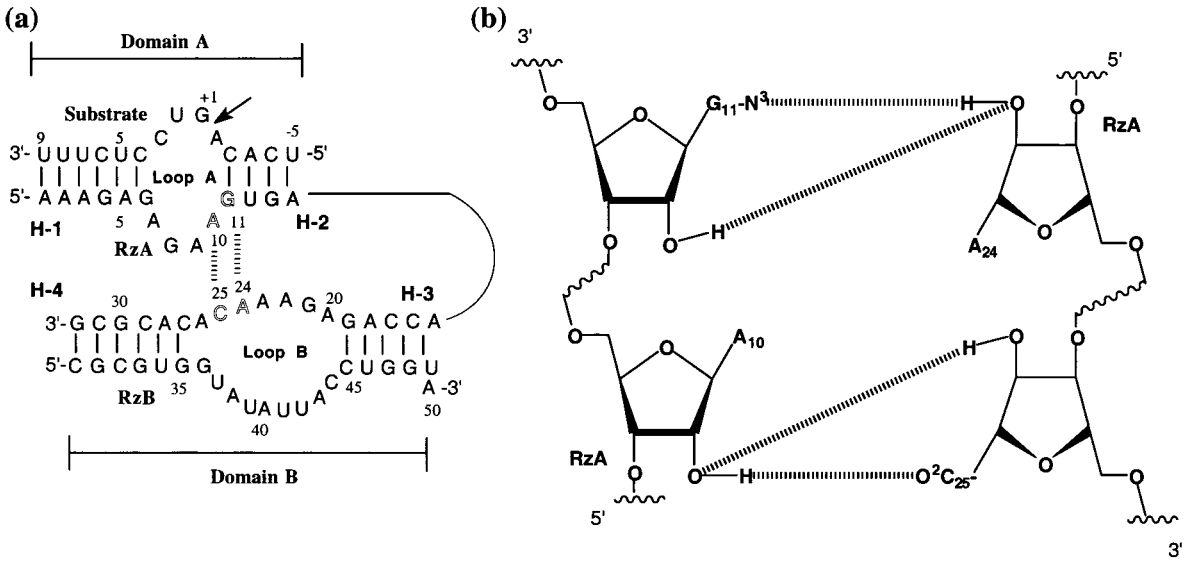

FIGURE 2: (a) Secondary structure of the 3-stranded hairpin ribozyme showing the four helixes H-1 to H-4. An arrow denotes the site of cleavage adjacent to the essential G_{+1} residue in the substrate strand. The outlined residues in the ribozyme A strand A_{10}, G_{11}, A_{24}, and C_{25} and dashed lines represent interactions proposed to be involved in a ribose zipper motif (*10*). (b) Schematic representation of the hydrogen-bonding interactions involved in the original proposal for a ribose zipper motif (*10*). (c) List of functional group modifications at the 2′-position used in this study and classification as potential hydrogen bond donors and acceptors. The role of 2′-F is shown as H-bond removal, although there is the potential for very weak hydrogen-bond acceptance and therefore this is shown in brackets.

overall reaction (k_{cat} = 0.2 min^{-1}) (*11*). Docking also has an important ionic requirement that is satisfied for the 4-way junction by micromolar concentrations of divalent metal ions such as calcium, magnesium, and manganese (*12*). The

minimal 2-domain hairpin requires a higher concentration of divalent metal ions for docking, but it can nevertheless achieve a high level and rate of formation of the docked configuration with a wide range of ions including cobalt (III) hexammine (*11*). Recent time-resolved FRET analysis has shown that docking requires specific interdomain interactions (*13*).

Steps in the minimum mechanistic pathway for cleavage of the minimal hairpin ribozyme include substrate association, docking, chemical cleavage, and product dissociation (Figure 1). Substrate association is extremely rapid (k_{on} in the region of 2×10^8 M^{-1} min^{-1}) if the substrate sequence is modified to avoid self-complementarity, and product dissociation is not rate limiting if the arms of the ribozyme are kept short (*14*−*16*). Neither step is proposed to be ion dependent. By contrast, both docking and the overall reaction have an essential ionic requirement, but the cleavage reaction is only efficient with a narrower range of ions. For example, among divalent metal ions, manganese is a poor inducer of hairpin ribozyme cleavage (*17*), but is highly effective in docking (*11*). It had therefore been expected that there might be an additional requirement for metal ions in the cleavage step, which is the case for some other small ribozymes such as the hammerhead. However, in the cleavage reaction of the hairpin, the evidence does not support a direct catalytic role for metal ions in cleavage. This evidence includes a shallow linear pH profile and no effect on the cleavage rate of thio-substitution of a nonbridging oxygen atom at the scissile phosphodiester, and cobalt(III) hexammine is particularly efficient at supporting hairpin ribozyme cleavage even though it cannot form inner sphere coordination complexes (*18*−*20*).

We showed recently that efficient hairpin ribozyme cleavage can take place in the absence of divalent metal ions when certain aminoglycosides or polyamines such as spermine are present (*21*). Indeed, the cleavage rate in the presence of 10 mM spermine at pH 7.5, 37 °C under single-turnover conditions was the same as in the presence of 10 mM Mg^{2+}. Very high concentrations (4 M) of monovalent ions such as ammonium also support hairpin ribozyme cleavage (*22*). These results provide further evidence that ions play only structural rather than direct catalytic roles in hairpin cleavage.

The most obvious way of rationalizing the ionic dependence of hairpin ribozyme cleavage is that specific ions are required first in domain docking and then subsequently in a second step just prior to chemical cleavage, most likely a conformational rearrangement, and that this second step may be rate limiting (Figure 1). A requirement for a conformational change that involves essential ions has been proposed in the cases of other ribozymes such as the hammerhead. The first indication was that major discrepancies were found between the nucleotide functional group requirements for hammerhead cleavage, as determined by site-directed nucleotide analogue incorporation (*23*), and structures of the hammerhead obtained by crystallography (*24*, *25*). The former technique assessed predominantly transition state functional group requirements while the latter provided information on ground-state functional group contacts. Second, significant localized changes in RNA conformation as well metal ion relocations were observed within the hammerhead catalytic core by crystallography techniques involving the freeze-trapping of proposed cleavage intermediates (*26*). Third, a strong case has been made recently for a major conformational rearrangement of the hammerhead in the transition state that involves relocation of an important ground-state magnesium ion that is distant to the cleavage site into proximity of the cleaved phosphodiester (*27*).

In the absence of a crystallographic or NMR model of the hairpin ribozyme, structural information regarding the docked conformation and possible subsequent conformational rearrangement has relied upon development of molecular models based on a range of other techniques. Our first model of the docked configuration was guided by available NMR results of loop A (*5*) and chemical probing data on the two individual domains (*8*) and utilized constraints imposed by the effects of interdomain disulfide cross-linking on the cleavage reaction (*10*). The model included a motif within internal loop A containing a sheared G·A base pair that has similar geometry to one found in the hammerhead ribozyme, and an unusual tandem arrangement of sheared A·A base pairs within internal loop B that is similar in structure to ones in the J 4/5 region of the group I intron. However, the most striking prediction from the hairpin model building is a ribose zipper motif similar to one found in the *Tetrahymena thermophila* group I intron between the P4 and P6 domains (*28*). The hairpin ribose zipper is characterized by a pair of bifurcated hydrogen bonds between the 2′-hydroxyl groups of ribose residues, a pyrimidine O-2, and a purine N-3, all occurring within the two internal loop regions (Figure 2b).

Since the first proposal of this docked hairpin model, the predicted pattern of non-Watson−Crick base pairs in domain B was confirmed by the NMR structure of isolated domain B (*6*). Some minor conformational differences of certain individual residues in domain B were found not to affect the predicted docked model to a significant extent (Masquida and Westhof, personal communication). Further, the alignment of the two domains has been probed by other techniques such as the accessibility of the folded core to hydroxyl radical attack (*29*) and by interdomain photoaffinity cross-linking (*30*). These results confirmed the orientation and gross features of the predicted fold in the model but suggested that an even more intimate contact was likely.

The prediction of the ribose zipper motif in the docked complex was strongly influenced by previously published data showing significant effects on hairpin ribozyme cleavage of individual substitutions by either a 2′-deoxyribonucleoside or a 2′-*O*-methylribonucleoside at A_{10}, G_{11}, A_{24}, or C_{25} (*31*). More recently, FRET analysis has revealed that 2′-deoxynucleoside substitution at either G_{10} or A_{11} significantly impairs interdomain docking (*11*).

Functional group substitution experiments using modified nucleotides have been carried out widely for other positions of the hairpin ribozyme. Dramatic effects on hairpin cleavage observed upon replacement of G_{+1} by inosine in the substrate strand led first to a proposal that this residue is directly involved in catalysis (*32*). Subsequently, all the essential purine residues in each internal loop were investigated by

[1] Abbreviations: DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PAGE, polyacrylamide gel electrophoresis; TEAC, triethylammonium acetate; TCEP, Tris(carboxyethyl)phosphine.

N^7-deaza substitution and elimination of exocylic amino groups (*33*). This work, together with further substitutions by abasic residues and propyl linkers (*34*), led to the proposition that the arrangement of residues within internal loop B in the transition state differed considerably from that in the ground state. Further evidence for a structural rearrangement has come from the finding that there are significant effects upon cleavage for a range of modified pyrimidine residues incorporated into positions U_{41} and U_{42} in loop B in a manner inconsistent with the ground-state structural model (*35*). Very recently, conformational rearrangements have also been proposed to occur in loop A on the basis of the effects on hairpin cleavage of pyrimidine modifications at U_{+2} in the substrate strand (*36*).

Bearing in mind the central role of the proposed ribose zipper in the docking model of the hairpin ribozyme, it is important to determine whether such proposed contacts are present in the active form of the ribozyme and whether further evidence could be obtained for conformational rearrangement. We now report the kinetic analysis of a range of hairpin ribozymes in which 2′-modified nucleosides have been incorporated at key residues thought to participate in the ribose zipper. We show that although certain 2′-hydroxyl groups are essential for cleavage, our results do not support the proposal that a ribose zipper hydrogen-bonding pattern is present in the active structure of the hairpin. Further, we discuss our results in the light of very recent data obtained from a qualitative study of nucleotide analogue interference analysis (NAIM) of hairpin ribozyme cleavage, from which an alternative arrangement of the ribose zipper was proposed (*37*). Our results are also not wholly consistent with this alternative model. Our data suggest that a network of hydrogen bonding and other interactions that are more complex than the ribose zipper may be involved in the active hairpin configuration. In particular, we suggest that changes in the conformation of domain B residue C_{25} may play a crucial role in hairpin ribozyme cleavage.

EXPERIMENTAL PROCEDURES

Preparation of RNA Oligonucletides. Oligoribonucleotides were prepared by 1 μmol of solid-phase synthesis on a controlled pore glass support and purified by anion-exchange HPLC on a NucleoPac PA-100 column (Dionex, Surrey, U.K.) as previously described (*10*, *38*). Desalting was achieved via extensive dialysis against water.

The purity of each oligonucleotide was checked by 5′-end-labeling with γ-[^{32}P]ATP and T4 polynucleotide kinase (*39*) followed by electrophoresis on a 20% denaturing polyacrylamide gel (PAGE). The authenticity of each oligonucleotide was checked by MALDI-TOF mass spectrometry on a Perseptive Biosytems Voyager DE spectrometer essentially as previously described (*38*). For example, single substitution of a 2′-deoxynucleoside at A_{10} or A_{24} within ribozyme B strand gave *m*/*z* values found of 10 437 and 10 439, respectively (calculated 10 433), compared to the all ribo ribozyme B strand *m*/*z* of 10 455 (calculated 10 449), within experimental error consistent with expectation for loss of a single oxygen atom. Further confirmation of the presence of a 2′-deoxynucleoside at A_{10} or A_{24} within ribozyme B strand was obtained by the technique of partial alkaline hydrolysis of a ^{32}P-labeled sample as previously described (*55*) except that the subsequent acidic hydrolysis of the 2′-3′-cyclic phosphate was carried out with reduced acid (0.05 M HCl) to prevent depurination. The absence of a band in the hydrolysis ladder under PAGE confirmed the correct positioning of the 2′-deoxy residue.

Ribonucleoside phosphoramidites, 2′-*O*-methyladenosine, and 2′-*O*-acetyl 2′-arabinocytidine phosphoramidites were obtained from Glen Research (via Cambio, Cambridge, U.K.). 2′-Deoxynucleoside phosphoramidites were obtained from Cruachem (Glasgow, Scotland). 2′-Trifluoroacetylamino-2′-deoxycytidine phosphoramidite was provided by Wolfgang Pieken (Proligo, Hamburg, and Colorado) but is also now available from Glen Research. 2′-Acetyloxyaminoadenosine phosphoramidite was provided by Leo Beigelman (Ribozyme Pharmaceuticals Inc., CO) and 2′-deoxy-2′-fluoronucleoside phosphoramidites were synthesized at ISIS Pharmaceuticals, Carlsbad, by the procedures previously reported (*40*).

Synthesis of Oligoribonucleotides Containing 2′-Deoxy-2′-thiocytidine. 2′-Deoxy-2′-tritylthiocytidine phosphoramidite was synthesized and incorporated into the 32-mer oligoribonucleotide essentially as previously described (*41*). The oligonucleotide was cleaved from the solid support by treatment with concentrated ammonia/ethanol (3:1 v/v) at 55 °C for 16 h, evaporated to dryness and treated with 1 M tetrabutylammonium fluoride in tetrahydrofuran (Aldrich) for 24 h at room temperature in the dark. After neutralization with 0.1 M triethylammonium acetate (TEAC) solution, the tetrahydrofuran was removed by brief evaporation and the remaining aqueous solution desalted on a NAP-10 gel filtration column (Pharmacia) in aqueous solution. The product was purified by reversed-phase HPLC on an Ultrosphere ODS reversed-phase column (10 × 250 mm) with elution using gradients of acetonitrile in 0.1 M TEAC (pH 6.5). The S-trityl group causes a significant delay in elution time compared to untritylated oligonucleotide impurities. The peak containing the desired 32-mer was collected and fractions checked by ^{32}P-labeling and PAGE analysis. The S-trityl oligonucleotide was converted into the corresponding S-pyridyl disulfide by treatment with silver nitrate solution followed by aldrithiol in 0.05 M Tris·HCl (pH 8.0) as previously reported (*41*). The product was separated once again by reversed-phase HPLC. The pyridyl disulfide product elutes earlier than the starting material. Formation of the disulfide derivative was checked by treating a sample of product 32-mer oligonucleotide with TCEP and carrying out analytical reversed-phase HPLC. A second peak identified by its retention time and UV spectrum as thiopyridone was seen only in the sample treated with TCEP (data not shown).

Determination of Ribozyme Kinetic Parameters. Kinetic parameters for cleavage of unmodified and modified ribozymes were determined under single- and multiple-turnover conditions as described previously (*10*) and data reported in Tables 1−3. For single-turnover experiments, separate solutions of ribozyme (equimolar mixture of RzA and RzB strands, 20−200 nM) in Tris·HCl (pH 7.5) and γ-^{32}P-labeled substrate RNAs (10 nM, 10 μL) in water were each incubated at 70 °C for 1 min and then cooled to room temperature over 15 min. The metal ion (usually magnesium) was added to its required concentration to the ribozyme solution and then both were incubated at 37 °C for 15 min. Reactions were initiated by mixing the ribozyme and

substrate solutions to give a final volume of 100 μL of 40 mM Tris·HCl. Aliquots (10 μL) were removed at six suitable time intervals, and the reactions quenched by addition to 10 μL of urea stop mix (7 M urea, 50 mM EDTA, 0.04% w/v xylene cyanol, and 0.04% w/v bromophenol blue). Samples were loaded on to a 20% denaturing polyacrylamide gel and subjected to electrophoresis at 12 W for 80 min. The resultant gels were dried and scanned using a phosphorImager (Molecular Dynamics, Buckinghamshire, U.K.), and the data processed using the program Image Quant (Molecular Dynamics) and quantitated by use of the Geltrak program as previously described (*10*). The initial velocities of the reactions (k_{obs}) at different ribozyme concentrations were determined from a plot of product formation against time, usually up to 30% of cleavage. Data from at least three independent experiments were obtained and usually fell within 20% of the mean. K_{obs} values were plotted against ribozyme concentration/k_{obs} using Eadie−Hofstee plots as described previously (*10*). Multiple turnover kinetic parameters were determined using substrate concentrations of 50−1000 nM and ribozyme concentrations of 1−80 nM as described previously (*33*, *34*).

pH Profiles. The pH dependencies of the k_{obs} of hairpin cleavage reactions were carried out under single- and multiple-turnover conditions described above. The following buffers were used to maintain pH: pH 4.8−5.5, potassium acetate/acetic acid; 5.5−6.7, potassium Mes (2-[*N*-morpholino]ethanesulfonic acid); 6.1−7.5, potassium pipes (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; 7.5−8.8, Tris·HCl; 8.6−10, potassium Ches (2-[*N*-cyclohexylamino]amino)-ethanesulfonic acid. The pH profiles were carried out under single- and multiple-turnover conditions for the unmodified, 2′-SH and 2′-NH_2 derivatives of C_{25} and the 2′-ONH_2 derivative of A_{10}, with 10 mM $MgCl_2$ and 40 mM buffer (data not shown). Experiments were carried out in triplicate or until high reproducibility was obtained (5−10% error).

Magnesium Ion Titration Assays. Ribozyme cleavage rates (k_{obs}) for the magnesium titration cleavage reaction for the 2′-deoxy derivatives of A_{10}, G_{11}, A_{24}, and C_{25} were determined under single- and multiple-turnover conditions as described above (Figure 3 and data not shown). The ribozyme and substrate concentrations (10 and 200 nM, respectively) were identical to those reported by Chowrira et al. (*31*). The titration experiments involved determination of the kinetic parameters at increasing magnesium ion concentration (varying from 0.1 to 150 mM) in the presence of a fixed concentration of Tris·HCl (40 mM, pH 7.5) at 37 °C. These experiments were repeated with an ion concentration fixed at 200 mM (total added magnesium and Tris·HCl was equal to 200 mM) (data not shown). Full single-turnover kinetics to derive k'_{cat} and K'_M were also undertaken at 10 and 100 mM $MgCl_2$ and 0.25 mM and 10 mM cobalt hexammine (data not shown).

Cleavage Reactions in the Presence of Other Metal Ions. Ribozyme cleavage rates (k_{obs}) for cleavage reactions with additional metal ions present were determined under single- and multiple-turnover conditions as described above with titrations of either $MnCl_2$ (freshly prepared) or $ZnCl_2$ in the presence of a fixed concentration of $MgCl_2$ or vice versa.

Cleavage Reactions Involving 32-mer Containing 2′-Deoxy-2′-thiocytidine. For the 2′-thio C_{25} ribozyme A strand, the pyridyl disulfide 32-mer was treated with varying concentrations of 10−100 mM DTT or 2-mercaptoethanol or TCEP (Strem Chemicals, Newburyport) for 60 min prior to the ribozyme cleavage assay. Varying concentrations of reducing agent were also maintained in the ribozyme reaction to ensure the free thiol was always present. To check for possible episulfide formation that would be followed by concomitant strand cleavage, the RzA strand containing the C_{25} pyridyl disulfide was 5′-^{32}P-labeled as described above. Various ions and reducing agents were then added to this labeled RzA strand that would mimic the reaction conditions used in the ribozyme cleavage assay, and aliquots of the reaction mixture were analyzed by PAGE. For example, after 24 h in the presence of 10 mM DTT, 10 mM $MgCl_2$, and 40 mM Tris·HCl, pH 7.5 at 37 °C, there was less than 10% degradation (data not shown). Reaction mixtures were also examined by MALDI-TOF mass spectrometry which showed a peak corresponding to the desired 2′-SH oligonucleotide was maintained during the incubation reactions (*m*/*e* 10 581, calculated; found 10 584 as potassium adduct), and no peak was observed for the corresponding oligonucleotide pyridyl disulfide.

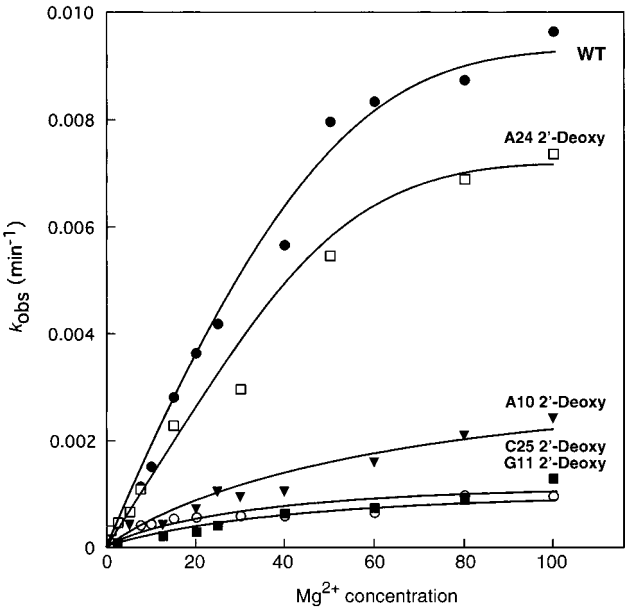

FIGURE 3: Graph showing the variation of observed rate constant with concentration of magnesium ions for the unmodified ribozyme (WT, filled circles) and 2′-deoxy analogues at A_{10}, (filled triangles), G_{11} (open circles), A_{24} (open boxes), and C_{25} (filled boxes).

RESULTS

In this work, we have utilized a three-stranded hairpin ribozyme construct that was first used by Chowrira et al. in studies of the 2′-hydroxyl requirements for hairpin cleavage (*31*). This ribozyme construct was used extensively in our previous studies of base and sugar functional group requirements within nucleotides in the internal bulge regions (*33*, *34*), as well as in our cross-linking studies (*10*) and in our studies of hairpin cleavage by nonmetallic ligands (*21*). We characterized the cleavage kinetics of this ribozyme under both single- and multiple-turnover conditions (*21*). An active ribozyme is prepared by annealing of three chemically synthesized oligoribonucleotides followed by incubation under the appropriate ionic conditions (Figure 2a).

2′-Deoxy and 2′-Deoxy-2′-fluoro Substitution Effects on Hairpin Ribozyme Cleavage. The four residues that are proposed to be involved in the ribose zipper are located in the same 32-mer oligoribonucleotide (ribozyme A strand, Figure 2a). Therefore, we carried out chemical synthesis of a series of 32-mers, each carrying a 2′-modified analogue with altered hydrogen-bonding capability at one of the four positions A_{10}, G_{11}, A_{24}, or C_{25}. Each of these oligonucleotides was then annealed in turn with the second unmodified ribozyme strand and then incubated with the substrate strand. The cleavage kinetics of these modified ribozymes under the appropriate ionic conditions could then be compared with that of the unmodified ribozymes. In consideration of the hydrogen-bonding pattern in the proposed ribose zipper (Figure 2b), a number of 2′-modifications would be expected to be informative. However, our studies were limited by the availability of suitable phosphoramidite reagents that could be used in solid-phase oligoribonucleotide synthesis. Potentially informative modifications are shown in Figure 2c.

Cleavage rate data have been reported under single-turnover conditions for both 2′-deoxy and 2′-*O*-methyl substitutions at each of the four positions under study (*31*). None of these substitutions was reported to affect substrate binding, showing that substitutions at these sites indeed report on subsequent steps in the ribozyme pathway. A 2′-deoxy substitution removes both hydrogen bond donating and accepting ability but has the disadvantage that the sugar conformation is C2′-endo, rather than C3′-endo, which is preferred generally by ribonucleotides. A 2′-*O*-methyl ribonucleoside adopts preferentially a C3′-endo configuration but is unable to act as a hydrogen bond donor. In principle, the oxygen atom at the 2′-position could serve as a hydrogen bond acceptor, but the bulk of the methyl group as well as the increased hydrophobicity makes acceptance of a proton greatly restricted in stereochemistry, and therefore, this substitution is less informative than might be supposed. This was shown recently in the case of probing of the ribose zipper of the *Tetrahymena* group I intron P4−P6 domain by substitution with 2′-deoxy and 2′-*O*-methyl analogues. Although there was a linear relationship between destabilization energy and the number of 2′-deoxy substitutions that removed individual hydrogen bonds, there was no such linear dependency in the case of 2′-*O*-methyl substitutions, believed to be due to the introduction of a hydrophobic element into the interactions (*42*).

There are also some discrepancies between our results and the data of Chowrira et al., in which the unmodified three-stranded ribozyme was reported to have a k'_{cat} value of 1.6 min^{-1} (*31*). By contrast, work carried out over several years in our laboratory has shown that this same three-stranded ribozyme consistently has a k'_{cat} value some 10-fold lower (*10*, *21*, *33*, *34*). Our slower cleavage rate was confirmed by subsequent work of Young et al. who also used the identical three-stranded ribozyme (*18*). Indeed no other hairpin ribozyme of similar sequence has been suggested to have a cleavage rate as fast as that reported by Chowrira et al. We therefore felt it was important to prepare the same 2′-deoxy analogues again for the four positions involved in the proposed ribose zipper and to remeasure their kinetics under single- and multiple-turnover conditions.

In addition, instead of the 2′-*O*-methyl analogues, we prepared the corresponding 2′-deoxy-2′-fluoro (2′-F) derivatives, which had not been studied previously in the context of the hairpin ribozyme. This analogue was introduced a few years ago into specific nucleoside positions in the hammerhead ribozyme both to probe the function of hydroxyl groups (*43*) and to increase endonuclease resistance (*44*, *45*). Very recently, a 2′-F derivative has been used as one of several analogues at position $U_{16.1}$ of the hammerhead ribozyme to show that observed hydrogen-bond donation by the 2′-hydroxyl group in the crystal structures is not essential for the cleavage reaction (*46*). Structurally, the 2′−F modification directs a sugar conformation that is about 90% C3′-endo and is similar to that for a ribonucleoside. A 2′-F substituent cannot act as a hydrogen bond donor, but it could in principle act as a very weak hydrogen bond acceptor. The existence of H···F bonds in aqueous solution has been questioned seriously (*47*), but there are others who believe that such bonds can indeed be formed, albeit weakly (*48*). It should be noted also that recent evidence of significant inter- and intramolecular O−H···F−C hydrogen bonding has been obtained in crystalline state and in alkane solution (*49*). We would suppose that, in aqueous solution, the effect of any H···F bonds would probably be extremely weak and unlikely to contribute much energy, if any, to the interaction.

Table 1 shows the effects of individual substitutions of 2′-deoxy and 2′-F at each of the four positions under both single- and multiple-turnover conditions and a comparison with the rates of cleavage of the unmodified ribozyme (Table 1). For the 2′-deoxy substitutions, K_M values did not differ from the unmodified ribozyme by more than a factor of 2 except for positions A_{24} and C_{25} under multiple-turnover conditions where K_M values were 5- and 8-fold increased, respectively. These results are generally consistent with data reported by Chowrira et al. (*31*). By contrast, k'_{cat} values under single turnover were 35- and 55-fold reduced for G_{11} and C_{25} respectively, but only 12-fold for A_{10} and 3-fold for A_{24}. Under multiple-turnover conditions, the rate reductions were mostly slightly larger than under single turnover, except for C_{25}. For three out of the four 2′-deoxy derivatives, the rate reductions were considerably lower than those reported by Chowrira et al. (230-fold for A_{10}, 160-fold for A_{24} and 320-fold for C_{25}) (*31*). Only at G_{11} was the rate reduction for 2′-deoxy substitution found to be similar (50-fold). In no case did we find the rate reduction greater than 50-fold.

For the 2′-F substituted hairpin ribozymes, again K'_M differences in values under single-turnover conditions were insignificant. By contrast, k'_{cat} values varied substantially between different positions of 2′-F substitution. For A_{10} and A_{24}, k'_{cat} was reduced only 5- and 3-fold respectively, whereas k'_{cat} was 100- and 470-fold reduced for G_{11} and C_{25}, respectively. Under multiple-turnover conditions, the results showed the same trends.

The results of 2′-deoxy and 2′-F substitution need to be considered in the context of the structure of the proposed ribose zipper (Figure 2b). At G_{11} and C_{25}, the 2′-hydroxyl groups are proposed to be hydrogen bond donors. Replacement by either 2′-deoxy or 2′-F, neither of which would allow hydrogen bond donation, results in a significant reduction in cleavage rate. The reductions in the case of 2′-deoxy substitutions are not as large as previously reported (*31*) but are nevertheless significant. The reductions in rate are even more pronounced in the case of the 2′-F substitutions. These results are therefore consistent with G_{11} and C_{25} hydroxyl

Table 1: Kinetic Data for the Cleavage of the Unmodified and Singly 2′-Deoxy and 2′-Fluoro Functionalized Positions A_{10} or G_{11} or A_{24} or C_{25} of the Hairpin Ribozyme RzA Strand under Single and Multiple Turnover Conditions

	position A_{10}			position G_{11}			position A_{24}			position C_{25}		
modification	k'_{cat} or k_{cat} (min^{-1})	K'_M or K_M (nM)	rate reduction[a]	k'_{cat} or k_{cat} (min^{-1})	K'_M or K_M (nM)	rate reduction[a]	k'_{cat} or k_{cat} (min^{-1})	K'_M or K_M (nM)	rate reduction[a]	k'_{cat} or k_{cat} (min^{-1})	K'_M or K_M (nM)	rate reduction[a]
unmodified												
single turnover	0.16 ± 0.01	94 ± 6		0.16 ± 0.01	94 ± 6		0.16 ± 0.01	94 ± 6		0.16 ± 0.01	94 ± 6	
multiple turnover	0.16 ± 0.02	26 ± 10		0.16 ± 0.02	26 ± 10		0.16 ± 0.02	26 ± 10		0.16 ± 0.02	26 ± 10	
2′-deoxy												
single turnover	0.013 ± 0.004	87 ± 38	12	0.0046 ± 0.0003	52 ± 9	35	0.059 ± 0.010	96 ± 23	3	0.0029 ± 0.0001	91 ± 7	55
multiple turnover	0.0046 ± 0.0003	49 ± 13	35	0.0032 ± 0.005	69 ± 36	50	0.012 ± 0.003	170 ± 35	13	0.0044 ± 0.000	275 ± 99	36
2′-F												
single turnover	0.034 ± 0.008	48 ± 23	5	0.0016 ± 0.0002	58 ± 11	100	0.056 ± 0.013	73 ± 26	3	0.000 35 ± 0.000 01	70 ± 9	470
multiple turnover	0.020 ± 0.002	119 ± 42	8	0.0018 ± 0.0008	289 ± 94	89	0.049 ± 0.009	330 ± 124	3	0.000 57 ± 0.000 12	113 ± 15	281

[a] k'_{cat} or k_{cat} rate reduction.

groups being involved in important hydrogen-bonding interactions in the active form of the ribozyme. The 2′-hydroxyl groups of A_{10} and A_{24} are proposed to be both hydrogen bond acceptors as well as donors (from the hydroxyl groups and the bases of G_{11} and C_{25}, respectively). However, in neither case does 2′-deoxy or 2′-F substitution have a large effect. In general, the rate reductions are all less than 10-fold. This implies that neither A_{10} nor A_{24} 2′-hydroxyl groups make a significant contribution to transition state stabilization, arguing against a role for either as hydrogen bond donors in the active form of the hairpin ribozyme. A_{24} is unlikely also to be a hydrogen bond acceptor.

Chowrira et al had stated also that inhibition of the cleavage rate by 2′-deoxy substitution at G_{11} and A_{24} (but not A_{10} and C_{25}) could be rescued by increasing magnesium concentration, suggesting that G_{11} and A_{24} 2′-hydroxyl groups play roles in binding magnesium ions in the transition state of the cleavage reaction (*31*). We have repeated these magnesium titration experiments for the four 2′-deoxy-substituted hairpins under multiple-turnover conditions using the conditions reported by Chowrira et al. (Figure 3). The unmodified ribozyme and the A_{24} 2′-deoxy analogue (which is only slightly impaired in cleavage rate) showed the expected dependence on magnesium concentration with a 10-fold increase in rate between 10 and 100 mM. In the cases of the other three 2′-deoxy analogues, a very small and gradual increase in cleavage rate was observed as magnesium concentration was increased. We did not observe for any of the 2′-deoxy hairpin analogues a "rescue curve" such as that reported by Chowrira et al., in which a sudden and large restoration of cleavage rate, approaching that of the unmodified ribozyme, was obtained at a higher magnesium ion concentration. Magnesium titrations carried out over a range of concentrations of substrate and ribozyme also failed to show any significant rescue (data not shown). We therefore find no evidence for a metal ion interaction with any of the four hydroxyl groups in question.

Effects of Modifications of the C_{25} 2′-Hydroxyl Group on Hairpin Ribozyme Cleavage. The data from 2′-deoxy and 2′-F substitution are consistent with the 2′-hydroxyl group at C_{25} playing a hydrogen-bonding role in the cleavage-active form of the ribozyme. We attempted to get further evidence for this role through use of three additional cytosine analogues modified at the 2′-position. These are the 2′-amino-2′-deoxy (2′-NH_2), 2′-deoxy-2′-thio (2′-SH), and 2′-arabino-nucleoside (2′-ara) cytosine analogues, all of which were available to us in a form suitable for phosphoramidite chemical synthesis.

The 2′-NH_2 analogue can function both as a hydrogen bond donor and as an acceptor. It was first introduced into the hammerhead ribozyme to probe hydroxyl group function (*43*, *44*). For example, in position $G_{10.1}$ of the hammerhead, a 2′-deoxy substitution caused more than 150-fold loss of activity, a 2′-F analogue was only 50-fold reduced in cleavage rate, and a 2′-NH_2 was only 15-fold reduced in rate (*43*). This result was taken as evidence for an important hydrogen-bonding role for $G_{10.1}$ 2′-hydroxyl group in the transition state of hammerhead cleavage. We therefore chemically synthesized a hairpin ribozyme carrying a 2′-NH_2 modification at position C_{25} and measured the cleavage kinetics under single- and and multiple-turnover conditions (Table 2). The results show that this modified ribozyme is only 8-fold reduced in cleavage rate under single-turnover and 17-fold reduced under multiple-turnover conditions. This rate is faster than that for either the 2′-deoxy or the 2′-F derivative and is therefore further evidence for hydrogen bond donation from the C_{25} 2′-hydroxyl group in the active form of the ribozyme.

Since a 2′-NH_2 nucleoside has a conformation that is about 75% C2′-endo, the conformational difference compared to a ribonucleoside was suggested in the case of the hammerhead ribozyme to explain why 2′-NH_2 substitution at $G_{10.1}$ did not fully restore the cleavage activity of the unmodified ribozyme (*43*). The lack of full restoration of the 2′-NH_2 analogue at C_{25} of the hairpin could also be due to such a conformational difference.

We next studied the 2′-deoxy-2′-thio modification at position C_{25} of the hairpin. A 2′-SH analogue has been incorporated chemically into oligonucleotides via phosphoramidite chemistry only recently (*41*). The synthesis involves the use of a trityl group for thiol protection during chain assembly and its removal at the end of synthesis using

Table 2: Kinetic Data for the Cleavage of the Unmodified and Singly 2′-Deoxy, 2′-Fluoro, 2′-Amino, 2′-Arabino, and 2′-Thio C_{25} Functionalized RzA Strand of the Hairpin Ribozyme under Single and Multiple Turnover Conditions

	single turnover			multiple turnover		
modification C_{25}	k'_{cat} (min^{-1})	K'_M (nM)	k'_{cat} reduction	k_{cat} (min^{-1})	K_M (nM)	k_{cat} reduction
2′-OH unmodified	0.16 ± 0.01	94 ± 6		0.16 ± 0.02	26 ± 10	
2′-deoxy	0.0029 ± 0.0001	91 ± 7	55	0.0044 ± 0.0009	275 ± 99	36
2′−F	$0.000\ 35 \pm 0.000\ 02$	70 ± 9	470	$0.000\ 57 \pm 0.000\ 12$	113 ± 15	280
2′-NH_2	0.021 ± 0.001	79 ± 9	8	0.0092 ± 0.0005	98 ± 17	17
2′-SH	$0.000\ 067 \pm 0.000\ 06$	100 ± 18	2390	<0.000 001	nd[a]	$\sim 10^5$
2′-arabino	<0.000 001	nd[a]	$\sim 10^5$	<0.000 001	nd[a]	$\sim 10^5$

[a] Not Determined.

aqueous silver nitrate solution. This is converted into the corresponding pyridyl disulfide by reaction with 2,2′-dipyridyl disulfide (*41*). To liberate the free 2′-SH derivative, excess reducing agent is added just prior to commencing the ribozyme cleavage reaction, which also prevents the possibility of formation of interstrand disulfides during the cleavage reaction. Another potential side-reaction is glycosylic bond cleavage via intramolecular C1′-C2′ episulfide formation, which can lead to chain scission at the modified linkage. In the case of the 2′-SH cytosine derivative, glycosylic bond cleavage at pH 7.5 was reported to have a half-life of 3000 h (*41*). Following incorporation of the 2′-SH cytosine derivative into the 32-mer oligoribonucleotide and standard 5′-^{32}P-labeling, we checked the stability of the 2′-SH oligonucleotide under standard ribozyme cleavage conditions (40 mM Tris·HCl, pH 7.5, and 10 mM $MgCl_2$, 37 °C) in the presence of 10 mM DTT and found by PAGE that there was less than 10% degradation in a 24 h period (data not shown).

The 32-mer containing a 2′-SH at position C_{25} was assembled into the complete hairpin, and the cleavage rate measured under single-turnover conditions (Table 2). The rate of cleavage was found to be 2500-fold slower than the unmodified ribozyme. Further, under multiple-turnover conditions, the rate was too slow to be measured. To check that the 2′-SH group was fully reduced by DTT under conditions of cleavage, we carried out the reaction in the presence of alternative reducing agents 2-mercaptoethanol and also TCEP, but no significant changes in cleavage rate were observed up to 100 mM in each case (data not shown). We also checked the reaction mixture by MALDI-TOF mass spectrometry for the presence of the correct 2′-SH oligonucleotide and found a peak at mass value 10 581 (calculated 10 584 as a potassium adduct), whereas no peak was seen in the mass area for the corresponding pyridyl disulfide (data not shown), from which the −SH group was derived. Since the pK_a of a 2′-thionucleoside is 7.3 (*50*), it is conceivable that the 2′-thiol might be insufficiently protonated to act as a hydrogen bond donor. Therefore, we carried out cleavage over a range of pHs between 4.8 and 9.0. Similar to that for the unmodified ribozyme, there was a reduced rate of cleavage at lower pH and a shallow linear pH dependence (data not shown). In addition, no rate enhancement was observed when manganese or zinc ions were added to the 10 mM magnesium ion concentration. Instead, the rate was reduced as manganese ion concentration increased, consistent with the absence of an important metal ion binding site near the C_{25} 2′-position. Note that disulfide formation can be facilitated in the presence of manganese ions (M.L.H., and J.A.P., manuscript in preparation), but we found that DTT concentration did not change cleavage rates, suggesting that disulfide formation was not a factor (data not shown).

The substantial reduction in cleavage rate for the 2′-SH derivative was not anticipated based on the results of the 2′-deoxy, 2′-F, and 2′-NH_2 derivatives described above. Since 2′-SH in a free nucleoside occurs as 80% C2′-endo (*51*), a reduction in cleavage rate might have been expected similar to that for the 2′-NH_2 C_{25} hairpin analogue. A further small reduction in rate might also have been expected since the hydrogen bond donation ability of a 2′-SH group is believed to be less strong than that of a 2′-hydroxyl group. Also, the sulfur atom is larger than oxygen, and this could add extra steric constraints that might hinder hydrogen bonding. However, the very large rate drop was unexpected and suggests that the 2′-SH modified hairpin may adopt an unusual conformation within the active ribozyme that is considerably different to that of the natural C_{25} residue within the unmodified hairpin. Perhaps the 2′-SH group is less able to participate in the interdomain interactions in the active form of the ribozyme and/or other functionalities in the active hairpin are affected by the unusual conformation, for example, the C_{25} base. The increased size of the −SH group compared to the −OH may also contribute to transition-state destabilization for steric reasons.

We further probed the conformational requirements of the C_{25} 2′-hydroxyl group by incorporating a 2′-arabinonucleoside (2′-ara), in which the hydroxyl group at C2′ is displaced 109° by rotation and where the sugar conformation shows a fairly equal distribution of C2′-endo and C3′-endo. Arabinonucleosides substituted into the hammerhead ribozyme have been remarkably informative. For example, a 10^5-fold slower cleavage rate was observed for 2′-ara G_5 compared to 1000-fold slower rates for the dG and 2′-F analogue (*52*). This showed not only the need for a hydrogen bond at this site but a precise directionality. An essential structural hydrogen bond was identified subsequently in the ground state of the hammerhead ribozyme between G_5 2′-hydroxyl and $C_{15.2}$ 2′-hydroxyl in stem III (*24*, *25*).

We therefore synthesized a 32-mer oligoribonucleotide containing a single arabinonucleoside at C_{25}, annealed this with the other unmodified RNA strands to produce the arabino-modified hairpin ribozyme, and measured the cleavage kinetics under single- and multiple-turnover conditions (Table 2). In each case, the cleavage activity was too low to be measured showing that the rate reduction was at least 10^5. This dramatic loss of cleavage activity is good evidence for the need for a precise orientation of the C_{25} 2′-hydroxyl group. Similar to the 2′-SH analogue, there must be an

Table 3: Kinetic Data for the Cleavage of the Unmodified, Singly and Doubly 2′-Functionalized A_{10} and/or C_{25} Positions of the RzA Strand of the Hairpin Ribozyme under Single and Multiple Turnover Conditions

	position A_{10}			position C_{25}		
modification	k'_{cat} or k_{cat} (min^{-1})	K'_M or K_M (nM)	k'_{cat} or k_{cat} reduction	k'_{cat} or k_{cat} (min^{-1})	K'_M or K_M (nM)	k'_{cat} or k_{cat} reduction
single modification	2′- ONH_2			2′- NH_2		
single turnover	0.0034 ± 0.0006	75 ± 25	47	0.021 ± 0.001	79 ± 9	8
multiple turnover	0.012 ± 0.0002	212 ± 9	13	0.0092 ± 0.0005	98 ± 17	17
double modification	2′-F			2′-NH_2		
single turnover	0.000 54 ± 0.000 08	98 ± 24	296	0.000 54 ± 0.000 08	98 ± 24	296
multiple turnover	0.000 33 ± 0.000 02	54 ± 17	485	0.000 33 ± 0.000 02	54 ± 17	485
double modification	2′-ONH_2			2′-F		
single turnover	0.000 22 ± 0.000 03	89 ± 24	727	0.000 22 ± 0.000 03	89 ± 24	727
multiple turnover	0.000 19 ± 0.000 06	124 ± 87	842	0.000 19 ± 0.000 06	124 ± 87	842

alternative conformation of the hairpin adopted by the 2′-ara C_{25} analogue that is unable to reach the active configuration.

Modifications at the C_{25} and A_{10} Hydroxyl Groups Are Inconsistent with Interribose Interactions. Neither 2′-deoxy nor 2′-F substitution at A_{10} was found to have a large effect on cleavage rate (Table 1). The slightly better cleavage rate for the 2′-F analogue could be due to a C3′-endo sugar conformation or possibly to a weak hydrogen-bonding interaction. To test further if hydrogen bonding at A_{10} was important at this site, we incorporated a 2′-oxyamino (2′-ONH_2) analogue at A_{10}. This analogue has the potential to be both a hydrogen bond donor and acceptor. In the hammerhead ribozyme, 2′-ONH_2 nucleoside incorporation led to enhancements of cleavage activity in some positions (U_7 and A_9), whereas at other positions ($A_{15.1}$), slower cleavage rates were found (*53*). Unlike the 2′-amino A analogue (which was not available to us), the sugar conformation of a 2′-ONH_2 nucleoside is expected to be predominantly C3′-endo due to the gauche effect of the 2′-oxygen atom.

The 2′-ONH_2 A_{10} modified hairpin ribozyme was found to have slower cleavage kinetics under both single- and multiple-turnover conditions than either the unmodified ribozyme or the corresponding 2′-F derivative (Table 3) and is similar to that for the 2′-deoxy derivative (Table 1). To rule out the possibility that the oxyamino group carries a positive charge at pH 7.5 that would prevent hydrogen bonding, we measured the cleavage rate for this analogue over the pH 4.8−9 range. The increase in cleavage rate between 7 and 9 was approximately 2-fold, very similar to that for the unmodified ribozyme. Thus, the results indicate that A_{10} 2′-hydroxyl group is unlikely to make an essential hydrogen-bonding interaction in the active form of the ribozyme.

The double substitution mutant hairpin with A_{10} 2′-ONH_2 and C_{25} 2′-F showed a very poor cleavage rate [over 700-fold slower than unmodified (Table 3)], which was no better than the C_{25} 2′-F analogue alone (470-fold reduced). Similarly, the double substitution of A_{10} 2′-F with C_{25} 2′-NH_2 also showed a poor cleavage rate (300−500-fold lower than unmodified and worse than the multiplied values of the single substitutions alone). Because of the ribose-like C3′-endo configurations of the 2′-F and 2′-ONH_2 analogues, these double substitution experiments would be expected to be more informative than corresponding double 2′-deoxy substitutions. These results showed that there was no compensation for rate losses that might have been expected if a weak F···H.N hydrogen bond between these two residues was present.

DISCUSSION

Ribose Zipper Motif. In the original ribose zipper proposal, which was modeled as an element in the ground-state docked complex of the two hairpin ribozyme domains (*10*), there are two sets of bifurcated hydrogen bonds involving residues G_{11} and A_{24} and also A_{10} and C_{25} (Figure 2b). Our data are inconsistent with these proposed interactions being present in the active form of the hairpin ribozyme that is cleaved. We found no evidence that hydrogen bonds are involved at either the hydroxyl groups of A_{10} nor A_{24}. However, our results are consistent with hydrogen-bond formation at the 2′-hydroxyl groups of G_{11} and C_{25}. None of the four hydroxyl groups tested appear to be in proximity to metal ion.

Recently, an alternative model for the hairpin ribose zipper was proposed based on a technique of Nucleotide Analogue Interference Mapping (NAIM) (*37*). The technique involves measurement of the ability of a range of nucleotide analogues tagged with a neighboring 5′-phosphorothioate and incorporated randomly into the hairpin to interfere with reverse ligation activity in a qualitative interference gel assay. 2′-Analogues used included the 5′-phosphorothioates of the four 2′-deoxynucleotides and the 2′-F derivatives of U, A, and 5-methylC. Strong interference was seen for A_{10} and C_{25} 2′-hydroxyl groups with both 2′-deoxy and 2′-F analogues and G_{11} with the 2′-deoxy analogue. Moderate interference was seen with 2′-F at A_{24}, but not with 2′-deoxy. From these and other results using base analogues, an alternative model for the ribose zipper was proposed (Figure 4). One interaction involves the 2′-hydroxyl groups of A_{10} and C_{25} and the N-3 of A_{10}. The other interaction involves the 2′-hydroxyl groups of G_{11} and A_{38} and the N-1 of A_{24}.

The Ryder and Strobel technique is a powerful mapping method that provides qualitative data about the requirements for functional groups in the active form of the hairpin ribozyme that is fit for cleavage reaction. The individual substitution method we have used also assesses these same requirements but provides quantitative kinetic data under different turnover conditions. Our 2′-deoxy and 2′-F results are consistent with those of Ryder and Strobel that the C_{25} and G_{11} 2′-hydroxyl groups are involved in a hydrogen-bonding interaction in the active hairpin whereas the A_{24} 2′-hydroxyl group is not. Ryder and Strobel also found strong interference at A_{10} for both the 2′-F and 2′-deoxy analogues.

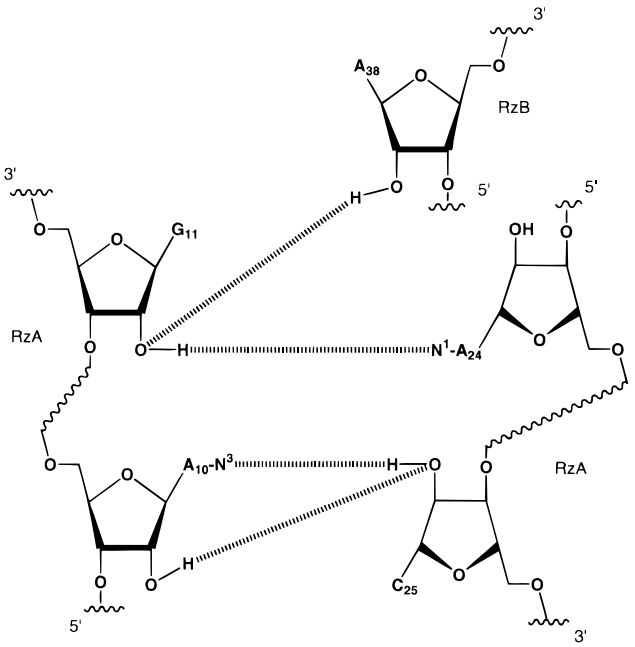

FIGURE 4: Schematic representation of the hydrogen bonding interactions in the revised proposal for a ribose zipper motif from Ryder and Strobel (*37*).

By contrast, we found only very modest effects at A_{10} (12-fold for 2′-deoxy and 5-fold for 2′-F under single-turnover conditions). In addition, we found that the 2′-ONH_2 substitution at this position also did not restore activity even though it has good potential hydrogen bond donor and acceptor capability. Therefore, the bulk of our evidence is not consistent with a hydrogen-bonding A_{10}:C_{25} interaction that was proposed in the Ryder and Strobel model (Figure 4). We previously measured the single-turnover kinetics of the 2′-deoxy analogue at position A_{38} and found a 50-fold reduction in cleavage activity (*34*). Therefore, this result taken together with our new data on G_{11} 2′-deoxy and 2′-F is consistent with the proposal of Ryder and Strobel for the second bifurcated interaction involving G_{11}, A_{38}, and A_{24}.

Data derived from functional group replacements and measurement of the resultant cleavage activity reported in this paper, in previous papers from our own laboratory (*33*, *34*) and from others (*35*, *36*), as well as the data from the NAIM technique reported by Ryder and Strobel (*37*), are all subject to important caveats. When an analogue incorporation into the hairpin ribozyme results in only a very small or no reduction in cleavage rate, then that ribozyme must be fit to carry out correct interdomain docking as well as any subsequent rearrangement or other event connected with the cleavage reaction itself. However, when analogue incorporation results in a significant deficiency in the cleavage activity, one must be aware that explanations other than a loss of intra- or intermolecular hydrogen bonding could be responsible for that deficiency. The two major likely alternative explanations are that (1) the functional group modification has affected the ability of the hairpin to form a docked complex and (2) a change of conformation of the individual nucleoside has taken place that affects the subsequent ability of the hairpin to reach the active configuration.

The only data available that addresses the first possible explanation, a docking deficiency, are reported by Walter et al., where the 2′-deoxy analogues of A_{10} and G_{11} were shown to be severely deficient in their abilities to dock as judged by FRET experiments (*11*). But the docking data also showed that other functionalities in the hairpin (such as the nature of the base and sugar at A_{-1}) also affect docking. It was suggested that the docked complex is stabilized by a number of weak interdomain interactions. Unfortunately, the use of FRET techniques to monitor docking of functionally modified hairpins requires complex RNA chemical synthesis involving simultaneous incorporation of two fluorescent labels as well as the modified nucleotide into the same oligonucleotide and also requires expertise and equipment only available in a few laboratories so far.

For some of our data where a dramatic deficiency in the cleavage ability was observed, such as for the 2′-ara derivative of C_{25}, it would be helpful to know if the analogue permits hairpin docking but results in an incorrect configuration that cannot reach the active configuration. We therefore investigated two other techniques that might report on hairpin-docking ability. In the case of the *Tetrahymena* group I intron P3/P4 domain, it was shown that docked and undocked states could be separated by native gel electrophoresis (*42*). We studied an uncleavable hairpin mutant containing 2′-*O*-methyl A_{-1}, which is known to have wild-type docking kinetics (*11*), and tried to observe native gel mobility differences between this ribozyme and ones containing one of a number of additional mutations reported to be docking deficient (such as A_{+1} or 2′-deoxy G_{11}). We used both our three-stranded ribozyme construct as well as a two-stranded hairpin, where the substrate is joined to residue A_{50} by a stretch of five cytidine residues (*9*), which is known to stabilize the docked conformation (*13*) (K. Hampel, personal communication). Under both published native gel conditions (*30*) and many others including high magnesium ion concentrations, we were unable to obtain gel resolution of docked and undocked states (data not shown). It is likely that in order to visualize any mobility differences reproducibly, more stably docked hairpin RNA constructs of different construction will be needed.

We also attempted to use Fe (II)-EDTA/hydrogen peroxide generation of hydroxyl radicals (*29*) to probe the differences in solvent accessibility of dockable and undockable hairpins using the same three-stranded and two-stranded constructs described above. Unfortunately, we were unable to obtain consistent and significant differences in gel patterns between the dockable and undockable hairpins for our hairpin sequences, despite many attempts (data not shown). Neither were significant differences observed in the CD spectra of the two types of hairpin (data not shown). In summary, we have been unable to generate data that address the possibility of defects in docking as an explanation for our modified ribozymes that are impaired in cleavage activity.

Role of Nucleoside C_{25} in Hairpin Cleavage. Analogues at C_{25} are particularly sensitive to 2′-modification. Almost all 2′-modifications at this position (2′-deoxy, 2′-F, 2′-SH, and 2′-ara) are strongly impaired in cleavage ability, some drastically so, whereas the 2′-NH_2 derivative is only modestly impaired. This site appears to be important in hairpin function partly through the need for hydrogen bond formation and partly for a conformational reason. C_{25} is an invariant nucleoside as judged by in vitro selection experiments (*7*),

and the base is moderately protected from reaction with dimethyl sulfate in the complex docked through addition of magnesium ions (*8*). In the ground-state NMR model of isolated domain B, C_{25} pairs with U_{37} through a single hydrogen bond (*6*). Replacement of C_{25} in the full hairpin ribozyme by an analogue missing the exocyclic amino group resulted in 20-fold loss of cleavage efficiency, consistent with loss of one hydrogen bond to an uncharged partner (*35*).

A very recent study has utilized molecular modeling, docking, and complementary base mutation of C_{25} and the base flanking the cleavage site G_{+1}. It was proposed that C_{25} is involved in a base-triple with G_{+1} and A_9 that is formed in the step of rearrangement that follows hairpin docking (*54*). If such a base-triple occurs, it would be expected to be highly sensitive to the precise conformational positioning of C_{25}. Therefore, misalignments in docking or any difficulties in C_{25} being able to change its conformation following docking could be crucial to the overall cleavage fitness of the hairpin. It is very likely that the 2′-F, 2′-SH, and 2′-ara derivatives at C_{25} give rise either to misalignment of the docked structures or to conformations of this residue that are ill-equipped to rearrange to the base-triple structure. There is precedence for such an interpretation from the 2′-ara substitution results in certain positions of the hammerhead ribozyme, which were proposed to lock the ribozyme into a wrong conformation during folding (*52*). Therefore, we believe that our data is not inconsistent with the base-triple model as outlined by Pinard et al.

In conclusion, our results provide further evidence that the active form of the hairpin ribozyme that is fit for cleavage has a significantly different configuration than that of the initially docked conformation. It is possible that a form of ribose zipper may be present in the initially docked configuration, as some preliminary studies of docking characteristics of A_{10} and G_{11} 2′-deoxy analogues have shown (*11*). It will be necessary to carry out further tests of docking using other analogues to verify this. Alternatively, a ground-state crystal structure would be the most decisive test of interactions in the docked hairpin. However, our kinetic studies are inconsistent with the ribose zipper being present in the active form of the ribozyme that is cleaved and which is therefore likely to have undergone rearrangement, including perhaps formation of an interdomain base triple (*54*). It will be a considerable challenge in the future to find techniques that can probe the structure of the conformationally rearranged and active hairpin and to determine the roles that ions play in bringing this about.

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